

Effect of FADD expression during UVC carcinogenesis

Maja Radnić*, Lidija Kalinić, Igor Matić, Ana Ruso, Inga Marijanović, Ivana Furčić and Biserka Nagy

University of Zagreb, Faculty of Science, Department of Biology, Horvatovac 102a, HR-10 000 Zagreb, Croatia.

Abstract. To become a cancer cell, a cell must inactivate apoptosis in order to avoid dying. This model predicts that cancer cells are more resistant to DNA damaging agents because they have deactivated the apoptotic pathway. Apoptotic signal caused by UVC radiation triggers intrinsic or mitochondrial apoptotic pathway. Other apoptotic pathway is coupled directly to FADD (Fas-associating protein with death domain), an adaptor protein essential for mediating apoptosis (extrinsic or death receptor pathway). UVC could trigger this extrinsic pathway. Our initial effort was to develop dose-response relationships for survival and apoptosis as a function of UVC radiation using embryonic fibroblasts derived from FADD knockout mice and their genetic counterparts. We examined the effects of UVC exposure on the survival of FADD positive (FADD^{+/+}) and FADD negative (FADD^{-/-}) cells in a time- and dose-dependent manner. FADD^{+/+} and FADD^{-/-} cells were irradiated with UVC light (254nm) using a germicidal lamp. The culture media was drained before the irradiation and fresh media was added after. The UVC doses in our study were 50 J/m², 75 J/m² and 300 J/m². The cell proteins were isolated 24 and 48 hours after each UVC exposure. The poly (ADP-ribose) polymerase (PARP), 113kDa nuclear enzyme, is the first protein cleaved in fragments of 89 and 24kDa during apoptosis and this event was analysed by Western blotting using antibodies specific for PARP. The results indicated that FADD^{-/-} cells irradiated with a dose of 50 J/m² do not undergo apoptosis after 24 hours which was not case with FADD^{+/+} cells. FADD^{+/+} cells irradiated with 50 J/m², 75 J/m² and 300 J/m² UVC doses undergo apoptosis after 24 hours as well as after 48 hours. All other results for FADD^{-/-} cells (75 J/m² and 300 J/m² after 24 hours and 50 J/m², 75 J/m² and 300 J/m² after 48 hours) were identical as results for FADD^{+/+} cells. We established significant difference in apoptotic response after UVC radiation between FADD^{+/+} and FADD^{-/-} cells. As we suspected FADD protein is involved in the extrinsic apoptotic pathway caused by UVC radiation, although main apoptotic pathway caused by UVC radiation is intrinsic through mitochondria.

KEYWORDS: *apoptosis; UVC radiation; Fas-associating protein with death domain (FADD); poly (ADP-ribose) polymerase-1 (PARP).*

1. Introduction

Cell death or apoptosis is essential physiological process important in many facets of normal physiology in animal species, including normal growth and fetal development or metamorphosis, regulation of cell turnover and tissue homeostasis and as a strategy for immune cell education against invading pathogens. Deregulation of apoptosis or defects in the regulation of tissue homeostasis giving rise to multiple diseases associated with either inappropriate cell loss or pathological cell accumulation, including cancer, neuro-degenerative diseases, autoimmune diseases and diabetes. Although several stimuli can induce apoptosis, little is known about the intermediate signaling events, including inhibition that connect the apoptotic signal to a common cell death pathway conserved across many species. Major advances have been made in understanding the signaling pathways mediated by the tumor necrosis factor receptor (TNFR) family in signaling apoptosis. Two cell surface cytokine receptors of the TNFR family, TNFR-1 and CD95 (Fas/APO-1), act as death receptors and a number of binding proteins have been identified which mediate apoptosis through these receptors. FADD (Fas-associating protein with death domain) or MORT1, (mediator of receptor induced toxicity) is a protein which interacts with the cytoplasmic domain of Fas/APO-1 acting as a downstream effector in the process of apoptosis [1]. Binding of Fas or TNF to their corresponding receptors leads to formation of a death-inducing signaling complex (DISC), composed of the adaptor protein FADD (MORT1) and procaspase-8, resulting in release of active caspase-8 [2-3]. Activated caspase-8 then triggers a cascade of different downstream caspases, finally resulting in apoptotic cell death. FADD is essential for TNF-induced apoptosis and has been shown to mediate TNF-dependent

* Presenting author, E-mail: mradnic@zg.biol.pmf.hr

activation of acid sphingomyelinase (A-SMase) [4], lipopolysaccharide-induced apoptosis [5], embryonic development [6] and T-cell activation and development [7-8]. UV (ultraviolet) light targets both membrane receptor and nuclear DNA, thus evoking signals triggering apoptosis. Apoptosis induced by UVC light has been proposed to be a consequence of receptor activation [9]. This conclusion was based mainly on the finding that UVC provokes clustering of CD95R /FasR [10]. In previous study, a gene-knockout (k/o) approach was employed to address the role of FADD in PDT induced apoptosis [11]. Using the same genetic model, we assessed now the role of death receptor pathway in apoptosis after UV radiation.

2. Material and Methods

2.1. Materials

DME (Dulbecco's modified Eagle) medium, antibiotic-antimycotic solution and TRI reagent were purchased from Sigma. Fetal calf serum was from Gibco-BRL. TNF-alpha was obtained from Boehringer Mannheim.

UV exposure was done using Germicidal lamp at 254 nm (Upland, Ca) at a distance of 26cm and the dose was monitored using UV meter (Solar Light).

FADD cell lines were gift from Dr. W.C. Yeh, Toronto, Canada.

2.2. Methods

2.2.1. Cell culture

Cells were cultured at 37⁰C in a 5% CO₂ atmosphere with DMEM containing 10% fetal calf serum, 2mM glutamine, 4.5g/l glucose, 100units/ml penicillin, and 100µg/ml streptomycin. Cells were sub-cultured in every 3-4 days using Trypsin-EDTA.

2.2.2. UVC exposure to FADD cells

Confluent layers of FADD cells, grown in 10cm dishes, after aspiration of the medium, were exposed to different doses of UVC (50–350J/m²). After irradiation the conditioned medium was returned to the cells.

2.2.3. Survival

The metabolic activity of UVC-treated cells in comparison with untreated cells was determined by means of MTT assay. One to forty-four hours after treatment, MTT solution, 5mg/ml in PBS was added to the cultured medium to final concentration of 0.5mg/ml. After 2-6h of incubation, medium was aspirated. The cells were lysed and the blue redox product was dissolved by adding 0.1–1ml ethanol. The absorption at 540nm was determined; the 620nm background absorption was subtracted.

2.2.4. Determination and quantification of apoptosis

Nuclear apoptotic changes were determined by staining with the DNA-binding fluorochrome-bis-benzimide trihydrochloride (Hoechst 33342, Calbiochem). Cells were harvested by combining floating and adhering cells, washed twice in PBS, fixed for 10min in 2% formaldehyde/PBS at room temperature, permeabilized with PBS/0.2% Triton X-100 for 1 minute and stained in 1µg /ml Hoechst-33342 for 2 minutes. The percentage of cells displaying typical apoptotic nuclear morphology (crescent shaped condensed chromatin lining nuclear periphery; apoptotic bodies), referred to as the apoptotic index, was then assessed using the fluorescence microscope (Zeiss). Each time triple determinations were performed counting at least 100 cells.

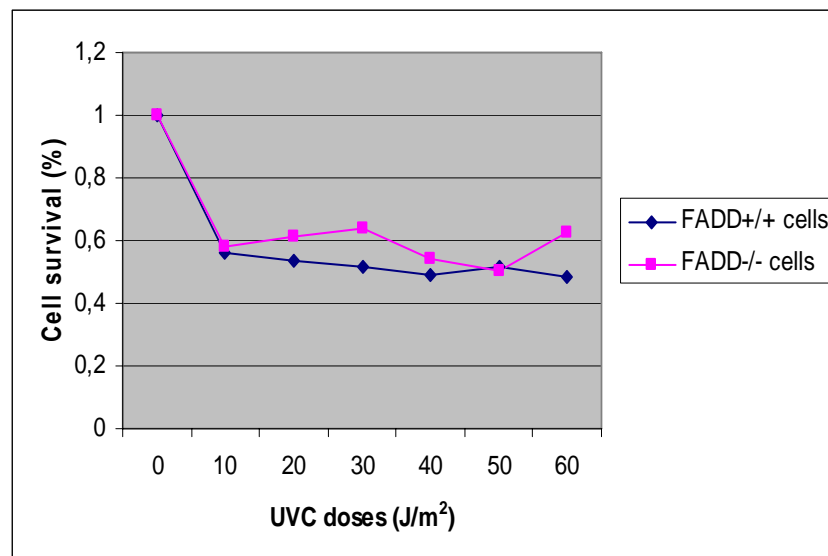
2.2.5. Preparation of cytosolic extracts and immunoblotting

Whole-cell extracts were prepared by lysis of PBS washed cells in 100 μ l loading buffer (62,5mM Tris-HCL, pH 6.8, 6M urea, 10% glycerol, 2% SDS, 0.003% bromphenol blue, and 5% mercaptoethanol (added freshly), homogenized and kept frozen at -20 $^{\circ}$ C until use. Cells extracts were heated at 65 $^{\circ}$ C for 5min. Protein content of samples was assessed by a Bradford assay (Bio Rad). Equal amounts (30 μ g) of protein were separated by 12 or 15% SDS-PAGE when PARP was analyzed. Separated proteins were transferred to PVDF transfer membranes (Hybond-P, Amersham) in transfer buffer (25mM Tris-HCL, pH 8.3, 192mM glycine, 20% methanol). Nonspecific binding was blocked by incubation in PBS containing 5% nonfat milk, 0.1% Tween-20 for 1h at room temperature. Anti-PARP antibody was diluted 1:1000 in PBS containing 1% nonfat milk, 0.1% Tween-20. After overnight incubation with agitation at 4 $^{\circ}$ C, the membranes were washed three times with 0.1% Tween-20 in PBS. Membranes were incubated with the secondary antibody, a horse radish peroxidase-labeled anti-mouse IgG antibody (Amersham), at the dilution 1:1000 in PBS containing 1% nonfat milk, 0.1% Tween-20 for 1h at room temperature. Membranes were washed four times with 0.1% Tween-20 in PBS. Proteins of interest were detected using the ECL Plus kit (Amersham) at room temperature for 1min. Western immunoblot images were visualized by a chemifluorescence procedure.

3. Results

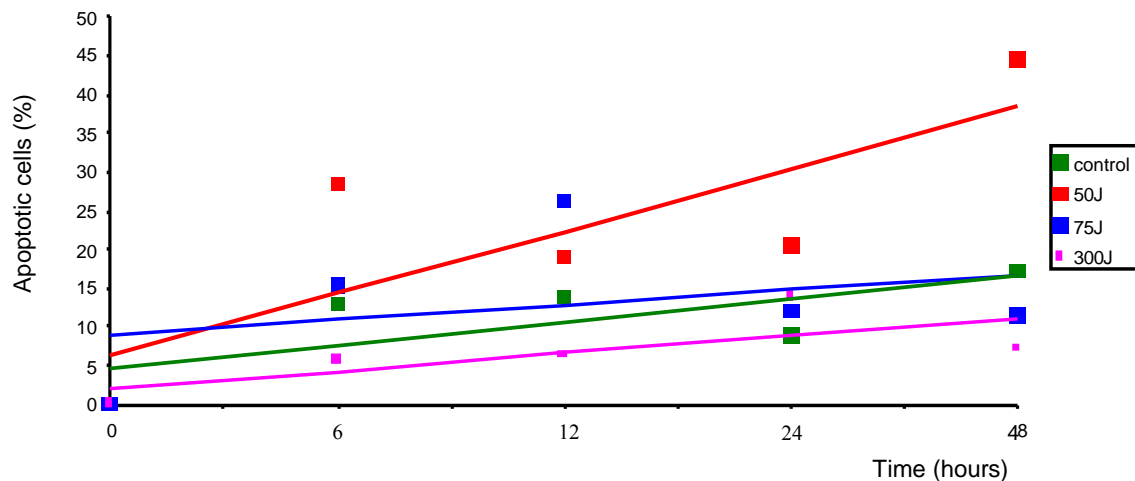
The survival of FADD knockout cells in comparison with the wild-type cells, determined by MTT assay after UVC irradiation is shown in Fig. 1. the results show that FADD knockout cells are less sensitive to higher UVC radiation dose.

Figure 1: Cell-survival response in FADD positive (FADD^{+/+}) and FADD negative (FADD^{-/-}) cells treated with 10, 20, 30, 40, 50, and 60 J/m² doses of UVC radiation



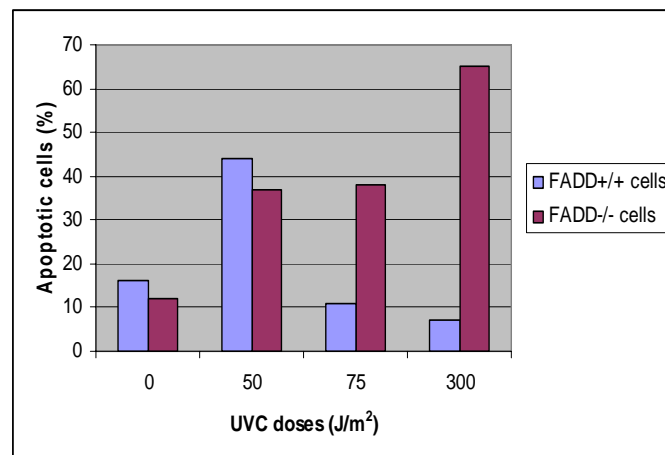
FADD^{-/-} cells lack adapter protein FADD, which is essential for recruiting and activating caspase-8, show sensitivity to UVC apoptotic stimuli.

Figure 2: Frequency of apoptosis of FADD negative (FADD^{-/-}) cells as a function of time. The apoptotic effect of UVC irradiation was tested after 50, 75, 300J/m². Apoptosis was measured after 6, 12, 24, and 48 hours. The results show that in time course fashion apoptotic DNA degradation in FADD^{-/-} cells started 6h after UVC light.



To address the question whether cell progression affects UVC induced apoptosis in both cell lines we increase the dose range up to 300 J/m² in both cell lines. After incubation for 48 hours, nuclear apoptotic changes were detected by staining with Hoechst 33342 using fluorescence microscopy. Frequency of apoptosis of FADD^{+/+} and FADD^{-/-} cells are presented in Fig. 3. With increase of cytotoxicity the apoptotic frequency decrease in FADD^{+/+} but not in FADD^{-/-} cells. The data show that UVC induced apoptosis by engagement of receptor pathway requires cell proliferation while the apoptosis resulting by enrollee other pathways rise with higher dose independently of cytotoxicity.

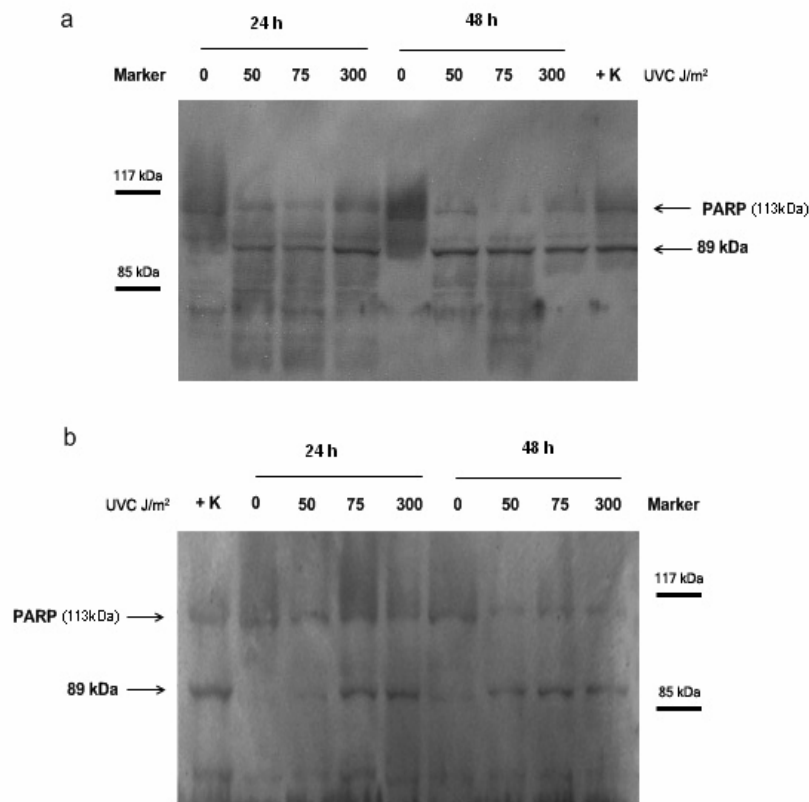
Figure 3: Correlation between portion of apoptotic cells and UVC doses in FADD^{+/+} and FADD^{-/-} cells after 48h of incubation.



Induction of PARP proteolysis by UVC radiation in FADD^{+/+} and FADD^{-/-} cells were assayed by Western blotting. PARP is the principal member of a family of enzymes possessing poly (ADP-ribosylation) catalytic capacity. It is conserved nuclear protein that binds rapidly and directly to both single and double strand breaks. Both processes activate the catalytic capacity of the enzyme. One of the early sign of programmed cell death is the caspase-induced poly (ADP-ribose) polymerase proteolysis. Thus it is crucial to investigate whether loss of FADD expression inhibits the PARP proteolysis after UVC radiation. Our results suggest that PARP cleavage by UVC light is equally efficient in FADD^{+/+} and FADD^{-/-} cells.

Figure 4: PARP cleavage in FADD^{+/+} and FADD^{-/-} cells as a marker of apoptosis. (a) FADD^{+/+} cells were exposed to UVC light (doses of 0, 50, 75 and 300 J/m²) and then lysed after 24 and 48 hours. Cell lysates were processed for Western Blot analysis using an anti-PARP antibody as described under Materials and methods. (b) FADD^{-/-} cells were exposed to UVC light (doses of 0, 50, 75 and 300 J/m²)

and then lysed after 24 and 48 hours. Mouse embryonic wild cells treated with TNF- α /Chx were used as a positive control (+ K) for PARP cleavage. PARP, a 113kDa nuclear enzyme, is cleaved in fragments of 89 and 24kDa during apoptosis



4. Discussion

The data show that UVC-induced apoptosis: a. do not require expression of FADD adapter protein; b. causes PARP proteolysis and caspase activation; c. causes different response on cell proliferation in FADD^{+/+} and FADD^{-/-} cells. Specifically, embryonic fibroblasts derived from FADD knockout (k/o) mice were used [6]. FADD (k/o) were exposed to UVC light. The survival and apoptosis were compared with those in wild type (w/t) fibroblasts. FADD knock out cells showed no significant inhibition of apoptosis induced by UVC radiation. The results suggest that expression of FADD interferes with proliferate signaling.

Corresponding with the cellular sensitivity determined by the MTT assay, Fadd^{-/-} cells displayed the highest level of apoptosis, whereas wt cells showed moderate apoptosis after UVC irradiation.

It is reasonable to conclude that apoptosis is a main cause of UVC-induced cytotoxicity, both in Fadd proficient and deficient cells. The data indicate that UVC light activates in both cell lines the Fas (CD95, Apo-1) receptor and the mitochondrial damage pathways. In Fadd^{-/-} cells, however, the high level of non-repaired DNA damage forces signaling by mitochondrial pathway up regulation, leading to enhanced UVC-induced apoptosis. Therefore, in Fadd^{-/-}, the mitochondrial pathway appeared to be dominant, while the receptor/ligand triggered pathway is blocked.

It was documented that UV directly activates death receptors in a ligand-independent way by inducing receptor clustering [12]. Activated death receptors trimerize and transduce the apoptotic signal via their intra-cytoplasmic death domain. UV induces the release of cytochrome c from mitochondria [13]. Whether UV affects the mitochondria directly or whether by receptor activation or the induced apoptosis is a consequence of DNA damage remains to be determined.

REFERENCES

- [1] CHINNAIYN, A.M., et al., "FADD, a Novel Death Domain-Containing Protein, Interacts with the Death Domain of Fas and Initiates Apoptosis", *Cell*, 81, 505-512, (1995).
- [2] BAKER, S.J and REDDY, E.P., "Modulation of life and death by the TNF receptor superfamily", *Oncogene*, 17, 3261-3270, (1998)
- [3] WALLACH, D., et al., "Tumor necrosis factor receptor and Fas signaling mechanisms", *Annual Review of Immunology*, 17, 331-367, (1999)
- [4] SCHWANDNER, R., et al., "TNF Receptor Death Domain-associated Proteins TRADD and FADD Signal Activation of Acid Sphingomyelinase", *J Biol Chem*, 273, 5916-5922, (1998)
- [5] CHOI, K.B., et al., "Lipopolysaccharide Mediates Endothelial Apoptosis by a FADD-dependent Pathway", *Biol Chem*, 273, 20185-20188, (1998)
- [6] Yeh, W.C., et al., "FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis", *Science*, 279, 1954-1958, (1998)
- [7] WALSH, et al., "A role for T cell activation and development", *Immunity*, 8, 439-449, (1998)
- [8] ZHANG, J., et al., "Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1", *Nature*, 392, 296-300, (1998)
- [9] SCHWARZ, T., et. al., "UV light affects cell membrane and cytoplasmic targets", *J Photochem Photobiol B*, 44, 91-96, (1998)
- [10] ARAGANE, Y., et al., "Ultraviolet light induces apoptosis via direct activation of CD95 (Fas/APO-1) independently of its ligand CD95L", *J Cell Biol*, 140, 171-182, (1998)
- [11] NAGY, B., et al., "Fumonisin B1 does not prevent apoptosis in A431 human epidermoid carcinoma cells after photosensitization with a silicon phthalocyanine", *Journal of Photochemistry and Photobiology B: Biology*, 57, 132-14, (2000)
- [12] YOUNG, A.R., "The sunburn cell", *Photodermatology*, 4, 127-134, (1987)
- [13] WOODCOCK, A. and MAGNUS, I. A., "The sunburn cell in mouse skin: preliminary quantitative studies on its. production", *Br. J. Dermatol.* 95, 459-468, (1976)